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Quantifying Recruitment of Cytosolic Peptides for HLA Class I Presentation: Impact of TAP Transport

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MHC class I ligands are recruited from the cytosolic peptide pool, whose size is likely to depend on the balance between peptide generation by the proteasome and peptide degradation by downstream peptidases. We asked what fraction of this pool is available for presentation, and how the size of this fraction is modulated by peptide affinity for the TAP transporters. A model epitope restricted by HLA-A2 and a series of epitope precursors with N-terminal extensions by single residues modifying TAP affinity were expressed in a system that allowed us to monitor and modulate cytosolic peptide copy numbers. We show that presentation varies strongly according to TAP affinities of the epitope precursors. The fraction of cytosolic peptides recruited for MHC presentation does not exceed 1% and is more than two logs lower for peptides with very low TAP affinities. Therefore, TAP affinity has a substantial impact on MHC class I Ag presentation. The Journal of Immunology, 2003, 170: 2977–2984.

Peptides derived by proteolytic breakdown of cellular proteins in the cytosol represent the overwhelming majority of MHC class I ligands (1). Therefore, the rate of production of an individual peptide in the cytosol, together with its transport into the endoplasmic reticulum, is likely to affect the efficiency of its presentation by class I molecules and its role in immune responses, and quantitative evaluation of cytosolic peptide processing and transport may be important for understanding epitope immunodominance and for vaccine design (2).

There is now substantial evidence that intracellular Ag processing plays an important role for epitope selection and immunodominance (reviewed in Refs. 1–3), and that many (probably the majority of) peptides with high binding affinities for MHC class I molecules are not provided by cellular Ag processing for class I presentation (4, 5). Numerous studies have provided clear evidence for an important role of the proteasome in selection of HLA class I ligands (reviewed in Ref. 6). Recent evidence suggests that peptide degradation by cytosolic peptidases limits peptide availability for class I presentation (7). Peptide transport by the human TAP is known to possess limited selectivity and may therefore contribute to epitope selection (8). Likewise, potential selectivity of N-terminal trimming of ligand precursors in the cytosol or endoplasmic reticulum (ER) could play a role in epitope selection (9–12). However, to date there is very little information on substrate selectivity of trimming or degradation peptidases.

Although a contribution of Ag processing to epitope selection has been clearly established, the vast majority of the pertinent studies have been qualitative and did not quantify the effect of variation in a given processing parameter on the efficiency of presentation of an epitope. As a result, very little is known about the overall efficiency of class I Ag processing, i.e., the number of source proteins that need to be degraded to produce one peptide/MHC complex for a peptide with a given rate of degradation, trimming, transport, and class I binding affinity. In a single study, the efficiency of processing of several epitopes from their source protein was determined. Pamer and colleagues (13) established that between 3 and 35 molecules of the source protein were required to generate one peptide/MHC complex. Considering that the vast majority of the proteasome output must be broken down to amino acids in order to be recycled for protein synthesis (7), recruitment of up to one-third of proteasome products for class I processing appears surprising. Moreover, theoretical considerations and estimates of cellular protein turnover suggest that the binding capacity of MHC class I molecules is very limited relative to the peptide output of the proteasome, and that a rate of one peptide/MHC complex per 10,000 degraded source proteins should be closer to the average efficiency of class I processing (14). Clearly, additional studies are required to establish the range of efficiencies with which cytosolic peptides are recruited for presentation by class I molecules.

We sought to establish the rate of recruitment of a model epitope for class I presentation. At the same time, we wanted to obtain a quantitative view on whether and how the human TAP transporters modulate the efficiency of class I processing. Although the principles governing peptide affinity for TAP are well understood, the impact of this selectivity on Ag presentation remains largely unknown. Human TAP transporters, while more permissive than their rodent counterparts, select peptides based on the residues in

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4 Abbreviations used in this paper: ER, endoplasmic reticulum; eGFP, enhanced green fluorescent protein; β₂m, β₂ microglobulin; tet, tetracycline.

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the three N-terminal positions and at the C terminus (15). The example of the rat, in which C-terminal selectivity of the nonpermissive TAP2<sup>+</sup> allele prevents peptide supply to certain class I allomorphs which are not naturally associated with this TAP allele, illustrates the dramatic potential impact of TAP on epitope selection (16, 17). However, evidence for a role of TAP selection in physiological settings is scarce. We have shown that extremely low TAP affinities of two viral peptides result in poor presentation; precursor peptides with higher TAP affinities were presented more efficiently (18). This study demonstrated that a minimal TAP affinity is required for peptide presentation. Evidence supporting this conclusion was recently reported by Brickner et al. (19) who identified a polymorphic peptide encoding a minor histocompatibility Ag, in which an N-terminal substitution reduced both TAP and class I binding affinity; presumably low TAP affinity precluded presentation of one of the two allelic peptide forms. These findings suggest strongly that TAP affinity needs to exceed a low threshold value for presentation; however, whether TAP transport of peptides with affinities above this threshold plays a role for the efficiency of Ag presentation is not clear.

This study was conducted with two objectives: 1) to define the proportion recruited for MHC class I presentation of a model peptide expressed in the cytosol; and 2) to establish the quantitative impact of TAP affinity on this rate. We report that only a small fraction of cytosolic peptides is available for Ag presentation, and that TAP affinity has a significant impact on epitope presentation.

**Materials and Methods**

**Peptides**

Peptides F10V (FLPSDFPPSV), R9L (RYRNASTEL), and S9L (SLYN TVATL) were purchased from Sigma-Genosys (Cambridge, U.K.) 95% pure. Precursors of F10V extended by single residues were synthesized with free amino acid termini using an Applied Biosystems SYNERGY peptide synthesizer (Foster City, CA) and F-moc chemistry. Purity was confirmed by analytical reverse-phase chromatography on a Vydac C18 column (Hesperia, CA) and, when necessary, synthetic peptides were purified by reverse-phase chromatography to >95% purity. Sequence and purity were confirmed also by mass spectrometry.

**Determination of HLA-A2 and TAP affinities**

TAP and HLA-A2 affinities were measured in competitive binding assays with radiolabeled reporter peptides R9L and F10V (6Y) used at 300 and 1700 nM, respectively, as described before (18). Results are expressed as inverse normalized IC<sub>50</sub> (1/IC<sub>50</sub> of test peptide/IC<sub>50</sub> of cold reporter peptide).

**Expression system**

An inducible system for coupled expression of peptides and a reporter protein was constructed by inserting an enhanced green fluorescent protein (eGFP)/ubiquitin cassette into the tetracycline (tet)-regulated expression plasmid MS4A (21) (a gift of F. Levy, Ludwig Institute for Cancer Research, Epalinges, Switzerland) with primers including a Nhel site (5′) and an Ncol site (3′). The resulting eGFP-ub cassette was transfected as a Nhel/Ncol fragment into the modified MS4A vector, resulting in plasmid MS4A-eGFP/ub. To insert peptide-encoding sequences, this plasmid was digested with NotI, followed by removal of protruding 5′ overhangs with mung bean nuclease (1.5 U/μg, 3 min at 37°C), and finally digested with Pael. Complementary oligonucleotides encoding peptides were designed to form blunt 5′ ends upon hybridization and contained a TAg stop codon and a Pael-complementary overhang at the 3′ end. In the resulting constructs, the first codon of peptides was directly preceded by the terminal Gly codon of ubiquitin. Correct sequences of the eGFP/Ub cassette and of peptide-encoding sequences were confirmed by sequencing. A construct with a single nucleotide deletion in the sequence encoding R-F10V was used as negative control in some experiments.

**Transfectants**

To study CTL recognition of synthetic peptides, murine TAP2-deficient RMA-S (22) or HLA class I-deficient human lymphoblastoid C1R (23) cells transfected with the class I molecule “HHD” were used. HHD consists of human β<sub>2</sub> microglobulin (β<sub>2</sub>m) linked to the N terminus of a chimeric class I H chain composed of the HLA-A*0201 el and α2 domains and the α3 transmembrane/cytosolic domains of H-2<sup>D</sup> (1). C1R-HHD transfectants were produced by electroporation of plasmid pBSK/HHD/neo; this plasmid is based on the published HHD expression plasmid (12) in which the neo gene encoding neomycin resistance inserted as SalI fragment. HHD expressing cells were selected with 0.5 mg/ml G418 (Life Technologies, Grand Island, NY).

eGFP/Ub/peptide constructs were expressed in C1R-HHD cells. Twenty micrograms of plasmid were electroporated (250 V and 960 μF) into 10<sup>6</sup> cells suspended in 0.25 ml of PBS with 10 mM HEPES (pH 7.4). Forty-eight hours after transfection, selection of transfectants with 0.5 mg/ml hygromycin (Life Technologies) was initiated. Transfectants were used for analysis by fluorometry and kill assays between 10 and 30 days after transfection. eGFP expression decreased steadily over time so that cells were discarded 4 wk after transfection. Experiments were performed with three independently produced sets of transfectants.

**Quantification of eGFP expression**

Expression of eGFP/Ub/peptide was induced by addition of doxycyclin (Sigma-Aldrich, St. Louis, MO) to cells cultured at 5 × 10<sup>4</sup>/ml 20 to 24 h before experiments; G418 and hygromycin were omitted from the induction medium. Based on preliminary experiments, doxycyclin concentrations of 0, 0.02, 0.08, and 0.6 μg/ml were used. eGFP expression was quantified both by flow cytometry and fluorometric determination of eGFP concentrations in cell lysates. For FACS analysis, cells were washed in PBS with 0.1% NaN<sub>3</sub> and 1% FCS and resuspended in the same buffer with 1 μg/ml propidium iodide (Sigma-Aldrich). eGFP expression by live cells excluding propidium iodide was then measured on a FACSCalibur machine (BD Biosciences, Mountain View, CA). For fluorometric assays, cells washed in PBS were lysed for 30 min at 4°C in PBS with 1% Nonidet P-40, debris was removed by centrifugation at 20,000 × g for 5 min. Then, cleared lysates were transferred into flat-bottom 96-well plates (Maxisorb, Nunc, Naperville, IL) and fluorescent light emitted at 510 nm was measured in a CytoFluor or Perkin Elmer (Bedford, MA) or a Jasco FP-750 fluorometer (Easton, MD). To establish a calibration curve and calculate the eGFP concentration in transfectant lysates, a Nonidet P-40 lysate of untransfected C1R-HHD cells was used to prepare a series of dilutions of recombinant eGFP (Clontech Laboratories, Palo Alto, CA). Using the m.w. of eGFP and Avagadro’s number, the number of eGFP molecules in the lysate was calculated. Finally, based on the number of cells lysed and the percentage of eGFP<sup>+</sup> cells in the FACS analysis, the number of eGFP molecules per cell was determined.

**Cytotoxicity assays**

Cytotoxic T cells were generated by peptide immunization of HHD mice, which express the single chain chimeric HLA-A2<sup>DR</sup> molecule described above on a D<sup>2</sup> and mouse β<sub>2</sub>m knockout background. Mice were injected s.c. at the base of the tail with 50 μg of peptide F10V mixed with 140 μg of helper peptide HBVcore 128–140 (TPAYRPNNAPIL) and emulsified in IFA (Difco, Detroit, MI). Eleven days later, spleen cells were prepared and restimulated with irradiated, peptide-pulsed (5 × 10<sup>4</sup> cells/ml, 10 μg/ml peptide, 2 h at room temperature in FCS-free RPMI 1640 medium) and LPS-induced HHD lymphoblasts. Cultured CTL lines were used on day 5 or 6 after restimulation in 4-h<sup>3</sup>H[TdR]-release assays. Specific lysis was calculated as follows: (experimental release – spontaneous release)/(total release – spontaneous release) × 100. Transfected target cells were analyzed for eGFP expression by flow cytometry on the day of the kill assay, and by fluorometric determination of eGFP concentration subsequently. Transfectant viability was in all cases >90%. To establish CTL recognition of synthetic precursors (see Fig. 5a), acid-stripped (see below) C1R-HHD cells were labeled with 51Cr in 1 h, washed twice and pulsed for 1 h at room temperature with peptides, washed once, counted, and used as targets in a 2.5-h<sup>3</sup>H[TdR]-release assay. One CTL line, and three CTL clones (clones 6, 7, and 11), all specific for peptide F10V, were used in the shown and discussed experiments. All CTL assays were performed at least three times.
Data obtained in kill assays with eGFP/Ub/peptide transfectants were processed to allow for comparison. First, data were adjusted as follows for percent eGFP^+ cells (as determined by FACS analysis): percent lysis/percent positive × 100. Corrected lysis data were then divided by the number of eGFP molecules per cell (expressed as multiples of 100,000) determined for the same transfectants by fluorometric assay (see Fig. 2, a and c). Data obtained were analyzed by nonlinear regression using Prism Graph software (Graphpad Software, San Diego, CA), and regression curves were used to determine lysis at 150,000 molecules eGFP/cell (see Fig. 4a). The correlation between TAP affinity and presentation efficiency (see Fig. 4c) was analyzed with Prism Graph software using the Spearman rank test.

Quantification of cell surface class I molecules and acid stripping

The number of HHD molecules on the surface of C1R-HHD transfectants was determined using a QIFIKIT kit (DAKO, Glostrup, Denmark) containing calibration beads coated to 3,600, 16,000, 53,000, 218,000, and 620,000 molecules of a mAb per unit. Beads were stained with the secondary Ab also used to stain C1R-HHD cells in the same experiment. Four experiments showed a linear relationship between the density of bead-coupled Ab and mean fluorescence (r = 0.999 or better); none of the experimental data points for the calibration beads deviated >15% from the calculated value on the linear regression line (not shown). In stainings with the HLA-A2-specific mAb BB7.2, C1R-HHD cells expressed ~42,000 HHD molecules on the cell surface; this contrasts to ~250,000 HLA-A2 molecules on homozygous lymphoblastoid Jethom cells (not shown).

Cell surface HHD molecules were stripped of peptides and reloaded with peptide F10V based on methods described by Engelhard and colleagues (24). C1R-HHD cells were suspended at 3 × 10^5/ml in 300 mM glycine (pH 2.5) with 1% BSA and incubated 3 min at room temperature. The buffer was neutralized by addition of 60 volumes of RPMI 1640 medium and removed by centrifugation. Then cells were suspended at 1 × 10^6/ml in RPMI 1640 supplemented with 5 μg/ml brefeldin A (Sigma-Aldrich), 6 μg/ml βm (Sigma-Aldrich), and peptide F10V (3 × 10^-10 to 1 × 10^-8 M), incubated for 2 h at room temperature, split in two halves and incubated another hour at 37°C. One-half of the cells were ^3Cr-labeled during the incubation at 37°C and then used for a cytotoxicity assay with mouse CTL specific for peptide F10V. Unabeled cells were stained for FACS analysis of HHD cell surface expression by subsequent incubations with the HLA-A2-specific mAb BB7.2 (American Type Culture Collection, Manassas, VA) and FITC-labeled goat Abs to mouse Ig (Southern Biotechnology Associates, Birmingham, AL).

Results

Experimental approach

Seeking to measure the efficiency of the processing steps downstream of degradation of source proteins by the proteasome, we expressed various “preprocessed” N-terminal-extended precursors of a single epitope in the cytosol. To avoid comparisons between epitopes recognized by different T cells, we used precursors of a single epitope extended by different single amino acids at the N terminus; in this system, peptides were likely to require N-terminal trimming for efficient T cell recognition. As minimal epitope, we selected peptide HBV core 18–27 (F10V), an HLA-A2-restricted immunodominant epitope (25) that, when expressed in the cytosol, is poorly presented due to its extremely low TAP affinity (18). We have previously shown that an F10V precursor extended by a single N-terminal residue that confers higher TAP affinity gives rise to more efficient presentation of F10V (18). Precursors that are presented more efficiently than the minimal epitope must escape cytosolic N-terminal peptide trimming because removal of the N-terminal residue by cytosolic peptidases leaves the poorly transported, and hence poorly presented, minimal epitope F10V. Peptide F10V was also suitable for this study because at least the previously studied F10V precursor transported into the ER is converted efficiently and quantitatively to the minimal peptide for presentation by HLA-A2 (18).

We obtained seven F10V precursor peptides extended by different single N-terminal residues and measured their TAP and HLA-A2 affinities in competitive binding assays (Fig. 1). All extensions increased TAP and reduced HLA-A2 affinity. In accordance with human TAP preferences, TAP affinities of precursors varied significantly and ranged from low to intermediate, with Arg and Asn conferring the highest, and Asp the lowest affinities (24-fold lower IC<sub>50</sub> for Arg than for Asp, Fig. 1a). HLA-A2 affinities of precursors varied moderately, with a 5-fold lower IC<sub>50</sub> for Asn than for Pro (Fig. 1b); however, all precursors had significantly lower A2 binding affinity than the minimal F10V epitope.

Expression system

To assess the quantitative impact of TAP affinity on peptide presentation, we chose a system that allowed us to establish the relationship between cytosolic copy number and T cell recognition for a peptide with a given TAP affinity (Fig. 2a). Peptides are expressed at the C-terminal end of a fusion protein also comprising eGFP and ubiquitin (21). eGFP allows for monitoring the percentage of eGFP/peptide-expressing cells by flow cytometry, and for determining the number of eGFP/peptide molecules per cell by fluorometric assays. The terminal Gly residue of ubiquitin immediately precedes the peptide (precursor) and is a signal for precise and quantitative peptide liberation because deubiquitinating enzymes cleave with high efficiency between the terminal Gly and all residues except Pro (26). Clonal variation due to genomic integration is minimized due to the episomal nature of the vector used. Finally, expression is regulated by tet so that copy numbers of the eGFP/Ub/peptide protein could be matched among transfectants by adjusting tet concentration (20).

Fig. 2b shows eGFP expression in two transfectants. Both lines show low level, but significant, eGFP expression in the absence of tet and dose-dependent induction of expression. Transfectants expressing P-F10V express higher levels of eGFP than those expressing R-F10V at all tet concentrations. Both eGFP expression in noninduced cells and dose-dependent induction of expression are observed in all transfectants, as shown in Fig. 2c. Optimal tet concentrations increase eGFP expression ~10-fold. Surprisingly, the level of eGFP expression varies considerably between the lines; for example, at all tet concentrations, mean fluorescence in the line expressing N-F10V is about twice that in the line expressing R-F10V. These differences were reproducible in the three independent sets of transfectants used in this study.
To take account of these differences in eGFP expression, transfectants used for CTL assays were evaluated with respect to two parameters: the percentage of eGFP-expressing cells, and the number of eGFP molecules per cell. Although the former parameter showed a slight variation (Fig. 2d), the latter varied strikingly. In the experiment shown, transfectants expressing P-F10V harbored a maximum of 2.6 \times 10^6 eGFP/peptide copies per cell but those expressing R-F10V only 1.1 \times 10^6 (Fig. 2e).

Comparing presentation efficiencies

To determine whether TAP affinity and other ER-associated parameters affected efficiency of presentation of F10V, we studied the killing of transfectants by CTL lines specific for this epitope. CTL were generated by immunization with peptide F10V of transgenic mice expressing a single chain m-HLA-A2.1/D b molecule. Due to the deletion of murine class I H and L chains, these mice mount more efficient A2-restricted CTL responses than traditional HLA class I-transgenic mice (22, 27). Use of CTL specific for F10V for testing transfectants expressing precursors was based on the assumption that precursor trimming to F10V was a prerequisite for stable HLA-A2 assembly and cell surface presentation. Although we have previously shown this assumption to be correct for precursor S-F10V (18), it was not clear whether the same applied to the other precursors. Therefore, we studied recognition of synthetic precursor peptides by a CTL line specific for F10V also used to test peptide presentation by transfectants (Fig. 2f). In contrast to peptide F10V, five precursors were recognized very poorly at a 3 \times 10^{-8} M concentration, while two others were not recognized at all. The latter included the precursor (Asn) presented most efficiently by transfectants (see below). Consequently, presentation of unmodified precursors by transfectants was clearly incompatible with efficient recognition of transfectants by CTL, and we concluded that all precursors were highly likely to be trimmed.

Lysis data corrected for the percentage of eGFP-expressing cells from two experiments with independent series of transfectants are shown in Fig. 3, a and b. As expected from the FACs analysis of eGFP expression, transfectants showed significant lysis even in the absence of tet and this increased in a tet dose-dependent fashion. In both experiments, lysis differed strongly between transfectants, with the highest levels for N-F10V and L-F10V, and the lowest for F10V and P-F10V.

To compare CTL recognition of transfectants directly, it was important to put cytotoxicity data in relation to the number of cytosolic eGFP/Ub/peptide copies in the target cells. This analysis showed that CTL recognition of some transfectants (R-F10V, N-F10V, and L-F10V) was high and close to maximal at low GFP/
Ub/peptide copy numbers, while that of others increased steadily with cytosolic peptide copy numbers (Fig. 3, c and d). This is almost certainly due to the fact that relatively low cytosolic concentrations of efficiently transported peptides are sufficient to load the number of MHC molecules sufficient for maximum CTL recognition. Thus, the minimal copy numbers obtained in our expression system were too high to observe a linear relationship between cytosolic copy number and CTL recognition for these efficient precursors. This limitation of our expression system (a factor of induction not exceeding 10 to 20 is typical for all regulated expression systems) may result in underestimating the true efficiency of presentation of the three “top” precursors (Asn, Arg, and Leu).

Fig. 4 illustrates the strong variation in processing efficiency observed when transfectants expressing identical numbers of cytosolic precursor or epitope copies were compared. Expression of 150,000 eGFP/Ub/peptide molecules resulted in 45% lysis for the most efficient precursors N-, R-, and L-F10V, contrasting with 8% for the least efficient peptides P-F10V and F10V (Fig. 4a). At

**FIGURE 3.** CTL recognition of, and eGFP expression by, transfectants. Two individual experiments with two independent series of transfectants and the CTL line specific for peptide F10V are shown in a–d; the left panels represent one experiment (E:T ratio 0.25), the right panels represent the other (E:T 4). Similar data were obtained in a third experiment with the same line, and in experiments with CTL clones 6 and 11. a and b, CTL recognition of transfectants. Data are expressed as percent killing of eGFP+ cells. In c and d, kill data also shown in a and b are plotted against the absolute number of eGFP molecules/cell.

**FIGURE 4.** Relative efficiencies of processing and presentation. a, Lysis of transfectants expressing equal numbers of molecules eGFP per cell. Specific lysis was plotted against the number of eGFP molecules and analyzed by nonlinear regression. Lysis at 150,000 (□) and 500,000 (■) molecules/cell was determined using the regression curve. Means ± SD of the two experiments shown in Fig. 3 performed with the CTL line and two independently generated series of transfectants are shown. b, Number of eGFP molecules required for 30% specific lysis. Data were obtained by regression analysis of the experiment shown in Fig. 3, a and c; the value for N-F10V is extrapolated and therefore a rough estimate. c, Relationship between TAP affinity (expressed as inverse IC50) and presentation efficiency (lysis at 150,000 molecules/cell, data from a). N-terminal extensions are indicated next to the corresponding data point.
500,000 copies/cell, differences were less pronounced but still significant. Fig. 4b offers another view of the tremendous differences in processing efficiency. Although cytosolic expression of $17 \times 10^7$ F10V or P-F10V molecules was required for 30% lysis, $0.7 \times 10^7$ R-F10V molecules (25-fold less) were sufficient for the same level of lysis.

Finally, we sought to establish whether or all of the striking differences in presentation efficiency were caused by distinct TAP affinities. Fig. 4c shows the relationship between TAP affinity and peptide presentation. The correlation between the two parameters was highly significant ($p = 0.0079$ in the Spearman test). Although the deviation of individual data points from an ideal line clearly must be interpreted with caution, the data for peptide F10V and four precursors are compatible with a linear relationship between the two parameters. In contrast, presentation of the Pro precursor may be less efficient than expected, while the opposite may be true for the Leu- and Asn-extended precursors. Poor presentation of the Pro precursor is likely due to inefficient peptide release in the cytosol, whereas other factors may be involved for the two other precursors (see below).

Estimating availability of cytosolic peptides for presentation to T cells

Having determined the number of cytosolic peptide copies required for CTL lysis, we sought to quantify the number of peptides presented by cell surface class I molecules. In preliminary experiments, we established that C1R-HHD transfectants expressed $\sim 42,000$ HLA-A2 molecules on the cell surface, and that the relationship between fluorescent Ab staining and Ag density was linear over a range of 3,600–620,000 Ab molecules (not shown). To quantify the number of F10V/HLA-A2 complexes, we adopted a technique suitable for estimation but not precise quantification of peptide/class I complexes on the cell surface (24). Surface class I molecules were first stripped of peptides by acid treatment and then loaded with graded concentrations of synthetic peptide F10V in the presence of brefeldin A which prevents export of new complexes formed in the cell. After incubation at 37°C to remove empty class I molecules from the cell surface, HLA-A2 expression is finally quantified by flow cytometry using a specific mAb. F10V-loading of HLA-A2 molecules is reflected in a shift of mean fluorescence and can be quantified based on the known number of HLA-A2 molecules per untreated cell (14). By using cells treated in the same fashion as targets in a CTL assay, an approximate relationship between the number of cell surface HLA-A2/F10V complexes and CTL recognition can be established.

Acid stripping reduced the density of cell surface HLA-A2 by 84%. Incubation with F10V resulted in dose-dependent reloading of these molecules (Fig. 5a). Incubation with as little as 10 nM peptide F10V led reproducibly to a detectable shift in fluorescence corresponding to formation of $\sim 210$ HLA-A2/F10V complexes, while loading with 30 nM peptide resulted in formation of 950 complexes (Fig. 5b). Target cells loaded in the same fashion gave rise to 45 and 59% specific lysis, respectively, by the CTL line (a) and of cells stained after incubation with various F10V concentrations. b, Specific killing by the CTL line (E:T of 2.5) of acid-stripped cells loaded with the indicated concentrations of F10V, together with an estimation of the number of F10V-loaded HLA-A2 molecules. Unloaded cells expressed 42,000 HHD molecules so that a fluorescence gain of one channel corresponded to loading of $\sim 105$ molecules. Therefore, $\Delta$ molecules HHD = $\Delta$ mean channel $\times 105$. Lysis data refer to the left y-axis, HHD expression to the right one.

For a numerical comparison of the different transfectants, we made the following assumptions: the data shown in Fig. 5 suggest that significantly $<210$ F10V/HLA-A2 molecules give rise to 30% specific killing. Assuming that 100 complexes are sufficient for 30% lysis, the ratio of cytosolic copies of F10V or F10V precursors to F10V/HLA-A2 complexes on the cell surface can be estimated on the basis of the data shown in Fig. 4b. According to this estimate, 500 (N-F10V) or 700 (R-F10V) copies of the most efficient precursors, 5,800 of a precursor with intermediate efficiency (Y-F10V), and 17,000 copies of the least efficient epitope forms (F10V, P-F10V) are required to produce a single F10V/HLA-A2 complex.

Discussion

This study reports two principal novel findings. First, it sheds light for the first time on the proportion of moderately to highly abundant cytosolic peptides that are available for class I presentation. Second, it suggests strongly that the affinity of an epitope precursor for the human TAP transporter is reflected proportionally in the efficiency of the presentation of the corresponding trimmed epitope by cellular class I molecules. However, in the case of some
epitopes, other factors in cellular Ag processing, including aminopeptidases in the ER, may have contributed to the observed differences in presentation efficiency.

Efficient release of peptides by deubiquitinating enzymes, but not the proteasome, in the cytosol of transfectants is a necessary (though alone not sufficient) prerequisite for interpreting differences in presentation efficiency as a result of TAP affinity. Given the enormous abundance of ubiquitin ($2 \times 10^6$ copies/cell), up to $2 \times 10^9$ fusion proteins should easily be handled by cellular deubiquitinating enzymes (14, 28). However, it has previously been described that deubiquitinating enzymes cleave poorly before Pro residues (26); therefore, the (poor) efficiency of presentation of the Pro precursor cannot be interpreted as a result of TAP transport. Although we are not aware of any evidence to this effect, it cannot entirely be ruled out that differential peptide release affected presentation of other precursors. Proteasome involvement in peptide generation in transfectants, for example by degradation of eGFP/Ub/peptide defective ribosomal products (29), was highly unlikely because 1) lactacystin did not affect presentation (data not shown), 2) only fully translated fusion proteins contain the epitope, and 3) the C-terminal end of such proteins should be a better substrate for deubiquitinating enzymes than for the proteasome.

Other factors not related to TAP transport may also affect peptide presentation, including peptide trimming in the cytosol and/or the ER. Cytosolic trimming has so far not been described to be selective. Preferred trimming of individual precursors would result in generation of the poorly presented F10V peptide or of shorter peptides unable to bind HLA-A2 and/or be recognized by CTL, and thereby result in a presentation efficiency below that expected according to TAP affinity. With the exception of P-F10V, all precursors were presented more efficiently than F10V. This observation suggests strongly that a sufficient proportion of all of these precursors escapes cytosolic trimming to the minimal epitope and further degradation and is trimmed only after TAP transport in the ER. Poorer than expected presentation of P-F10V was probably due to poor peptide release from ubiquitin, however, cytosolic trimming of this precursor to F10V, or its selective cytosolic degradation, cannot be ruled out.

Presentation of N-F10V and L-F10V may be more efficient than expected according to TAP affinity. At least four reasons are conceivable: preferred release from ubiquitin, resistance to cytosolic trimming, resistance to cytosolic and/or luminal degradation, and more efficient trimming in the ER. Although Shastri and colleagues (12, 30) have provided evidence against selectivity in luminal removal of the residue preceding the minimal epitope by the luminal peptidase ERA(A)P(1), recent studies suggest that the ER may contain up to four aminopeptidase activities, one or several of which may display selective trimming (31, 32).

This study demonstrates that the selectivity of human TAP transporters can have a strong and graded effect on class I presentation and should therefore be considered an important parameter in natural epitope selection. This is the first study to establish a quantitative relationship between TAP transport and HLA class I presentation. The studied range of TAP affinities extends from very low to intermediate and results in up to 34-fold differences in presentation efficiency. It is important to underline that low to intermediate TAP affinities are typical for HLA class I ligands while high affinities such as those of reporter peptides in TAP binding or transport assays are typical for ligands for a relatively small group of HLA class I molecules, notably HLA-B27 (33). It can be extrapolated that the latter peptides might be presented with significantly higher efficiency.

Poor recognition of synthetic precursor peptides by CTL specific for F10V suggested strongly that all precursors were trimmed in the ER for presentation by HLA-A2. The trimming requirement presumably reflects poor HLA-A2 binding of precursors that precludes stable assembly with HLA-A2 (Fig. 1b). Although we have recently shown that ER trimming was not rate-limiting for presentation of several HLA-A2 ligands (10), it cannot be ruled out that N-terminal peptide trimming limits the overall efficiency of presentation of other precursors. Both the length and sequence of some extensions may reduce limit efficiency.

Our study sheds light on the overall recruitment of cytosolic peptides for Ag presentation. The highest efficiency is attained for precursors N- and R-F10V, for which, at a cytosolic copy number of 150,000, ~500 to 700 cytosolic peptides give rise to formation of one peptide/HLA-A2 complex. This number rises to 5,000 for one complex for S-F10V with intermediate efficiency, and to 17,000 for peptides P-F10V and F10V. Due to the method used, these numbers are estimates and may deviate up to several-fold from the true numbers. Additional uncertainty is introduced by the fact that recombinant and intracellular eGFP molecules might have distinct specific activities. However, the estimates are useful because they define the order of magnitude of the processing efficiency of epitope F10V. Interestingly, for all peptides but P-F10V and F10V, the relative yield of Ag presentation, i.e., CTL recognition per cytosolic eGFP/Ub/peptide copy is much higher at low tet concentrations (Fig. 3, c and d). We suggest that this reflects the parabolic response profile of CTL: a small number of HLA/peptide complexes (frequently <100) is sufficient for significant target recognition by CTL, but full target lysis typically requires a much higher number of complexes (a few thousand in the case of this study).

Little published information on quantitative aspects of class I processing is available. In the report mentioned above, Pamer and colleagues (13) studied three epitopes derived from proteins of an intracellular bacterium and found that degradation of 3–35 source protein molecules was required to produce one peptide/MHC class I complex. Assuming that every degraded protein molecule gave rise to each of the studied peptides (a certain overestimation), Pamer’s numbers can be compared with ours. Although a ratio of 35:1 (degraded protein/prepared peptide) is not far from our data for precursors with intermediate TAP affinity, especially for lower cytosolic protein/peptide copy numbers (see above), a ratio of 3:1 appears as exceptionally efficient presentation. However, in light of our findings, such high efficiencies may be typical for peptides with high class I and TAP affinities; note that the TAP affinities of the F10V precursors studied here did not exceed intermediate levels. Another study is more difficult to compare with our data. Montoya and Del Val (34) reported a maximum efficiency of one peptide/class I complex for 3900 functional cytosolic copies of the source protein β-galactosidase. However, given that the rate of Ag degradation was unknown, and that defective ribosomal products, presumably the major source for many antigenic peptides (29), were not taken into account, the number of cytosolic peptide copies, and the percentage recruited for Ag presentation cannot be estimated from the data in that study.

Our data are compatible with recent considerations by Yewdell (35) who estimated that, on average, 10,000 copies of a 600-residue protein are degraded to produce one peptide/class I complex. Assuming that the protein contains about three ligands for an individual class I molecule, and that the proteasome produces one of these efficiently (14), a ratio of 10,000 cytosolic peptide copies for one peptide/class I complex can be deduced. This figure is within the lower range of our observations and suggests that, in a physiological setting, precursors like N-F10V or R-F10V may represent highly efficient epitope forms. Moreover, this accordance suggests that cytosolic peptides expressed in the eGFP/Ub system do not
differ significantly from proteasome products with respect to recruitment for class I presentation.

In conclusion, presentation by a class I molecule emerges as a marginal use even of cytosolic peptides that are well adapted to the downstream processing machinery. This finding presumably reflects the fact that cytosolic peptides are primarily broken down to individual amino acids and reused for protein synthesis. Multiple cytosolic peptidases with a possible involvement in peptide breakdown have been identified and partly proposed to limit peptide availability for class I presentation (7, 9, 36). We have demonstrated in this study that the efficiency of TAP transport has a strong effect on the size of the peptide pool that can be extracted from the cytosol for Ag presentation. Modulation or selectivity of other hypothetical cytosolic factors, such as peptide chaperones, ER-associated proteases, or proteasome modifiers, may similarly have dramatic effects on class I Ag presentation.

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References